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(54) Title: ASSAY FOR HEPATITIS C VIRUS HELICASE (NS3) RNA BINDING

(57) Abstract: The invention is directed to assays or kits containing and methods of using NS3 derived from hepatitis C virus protease/helicase that has been found to interact with the 3'-terminal sequence of viral positive-and negative-strand RNA. Assays, kits and methods directed to NS3's associated analogs in other viruses are also described herein. Methods of treatment of a subject having HCV infection are also described, by administration of an agent to the subject which inhibits the binding of helicase to HCV RNA.

FOR HEPATITIS C VIRUS HELICASE (NS3) RNA BINDING

FIELD OF THE INVENTION

The present invention relates to binding assay and methods for the measurement of Hepatitis C virus (HCV) helicase NS3 based on results from the studies of the interaction of HCV helicase NS3 with the 3'-terminal sequences of viral positive- and negative-strand RNA.

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) is the primary causative agent of parenterally transmited non-A, non-B hepatitis and affects a significant part of the world wide population. HCV infection frequently leads to chronic hepatitis, cirrhosis of the liver and possibly hepatocellular carcinoma (11, 45). The HCV, a member of the *Flaviviradae* family (44), has been a difficult virus to study due to the lack of an appropriate tissue culture system and an adequate, simple and low-cost animal model. The RNA genome of HCV has recently been cloned and the single stranded, plus polarity RNA genome of the virus, is approximately 9500 nucleotides long flanked by untranslated region (UTR) at both 5' and 3' ends (22, 28, 48). The 5' UTR of HCV RNA (341 nucleotides) is highly structured and contains an internal ribosome entry site (IRES) which extends to nucleotide 370 partially overlapping with the structural protein (core) coding sequences (8, 20, 21, 51, 53). The secondary structure of the 5' UTR appears to be highly conserved amongst various HCV strains and similarity in the structure with members of the pestivirus family has been reported using biochemical approaches (7, 22). The 5' UTR of the viral RNA is followed by a single large open reading frame that encodes a polyprotein of approximately 3,000 amino acids which is subsequently proteolytically processed by cellular signal peptidases and two HCV encoded proteases to produce mature structural and functional proteins (16-18). The 3' untranslated region (3' UTR) of approximately 200 nucleotides contains three distinct regions – a short region with sequence heterogeniety preceeding a poly (U/UC) region of variable length followed by a highly conserved sequence (X region) of approximately 100 nucleotides (6, 24, 30, 48, 55). This X region of conserved sequence forms three stable stem loop structures, SL-I, SL-II and SL-III. The major non structural

proteins include two proteases NS2 and NS3 followed by NS4A, NS4B, NS5A and NS5B (12). NS4A, a 54 amino acid polypeptide however, seems to act as a cofactor for NS3 activity and the central domain has been implicated as essential for this role of NS4A (3, 4, 15, 33, 34, 49). Almost nothing is known about HCV RNA replication except that various laboratories of late have shown synthesis of full length complementary RNA (and dimeric RNA) which can be achieved *in vitro* by the NS5B protein (38, 40, 60).

HCV NS3 protein has been the subject of intense study due to its associated protease and helicase activities. The C terminal 450 amino acids of NS3 protein constitute a polynucleotides-stimulated NTPase activity (25, 42, 46), 3'-5' unwinding activity (19, 25, 47) and a single stranded RNA binding activity (19, 47). The N-terminal one third of NS3 contains a serine protease activity and is responsible for the down stream cleavages in the non structural region (31, 50). The three dimensional structure of NS3 by X ray crystallography has been solved and structure based mutagenesis of NS3 has identified important amino acids residues required for helicase and ATPase activities (9, 29, 37, 59). Recent studies have shown that co-expression of NS4A protein directs NS3 protein to the endoplasmic reticulum which otherwise is diffusely distributed in the cytoplasm and nucleus in the absence of NS4A (54).

Many single stranded positive strand RNA viruses encode their own helicases (and NTPases). They are thought to play a role in viral RNA replication. Previous work has shown that the poliovirus encoded NTPase (and helicase) 2C, specifically interacts with the 3' terminal sequences of viral negative strand RNA (2). Apart from its specific RNA binding ability it is also able to interact with cellular cytoplasmic membranes (5, 10, 13, 14). Since poliovirus RNA synthesis takes place in cytoplasmic membranes, it is believed that the poliovirus 2C protein anchors the negative strand to the cytoplasmic membrane thus allowing initiation of positive strand RNA synthesis to occur. Moreover, initiation of positive strand synthesis is likely to require an unwinding activity to melt the

double stranded structure at the 3' end of negative strand formed by initial copying of the input viral plus strand RNA.

Thus, a need exists for further examination of the interaction of intact NS3 and the ΔpNS3 (with helicase domain only) with the 3' terminal sequence of negative strand RNA of HCV and the applications of NS3 binding to the 3' terminal sequence of viral positive-and negative-strand RNA in HCV RNA replication. The present invention shows that both NS3 and ΔpNS3 interact specifically with the 3' terminal sequences of HCV negative strand RNA. This interaction is impaired by deletion of the 3' terminal sequences of the negative strand. The present invention also provides a more detailed analysis, which suggests that a stem loop structure within the 3' terminal sequence is important for interaction with NS3. Unlike the other viral encoded helicase (like the 2C of poliovirus) which interacts with the 3' UTR of the negative strand but not with the 3' sequences of the positive strand, NS3 (and ΔpNS3) interacts with both the positive and negative strand 3' UTR sequence. Thus, the present invention shows that NS3 specifically binds to the 3' ends of both positive and negative strand RNAs of HCV.

SUMMARY OF THE INVENTION

The present invention is directed in one aspect to a method for assaying a sample suspected of containing hepatitis C viral (HVC) RNA comprising: contacting the sample with an NS3 protein, fragment, or mutant thereof to form a nucleoprotein complex; and measuring the amount or presence of the HCV RNA in the sample. In a preferred embodiment, the NS3 comprises a label.

In another related aspect, the invention is directed to kit for assaying a sample suspected of containing a HCV RNA, wherein the kit comprises a composition having an NS3 protein, fragment or mutant. The kit can be used as an assay to measure the presence or amount of HCV RNA in a sample. Preferably, the NS3 comprises a label. In a preferred embodiment, the kit further comprises instructions for assaying the sample.

The NS3 protein could be *in vivo* expressed using either an prokaryotic or an eukaryotic expression system including insect cells. Furthermore the protein is purified using affinity chromatography or conventional protein purification from the cell lysate. as is common in the art The protein may be either full length or partially truncated. The truncated protein can have a stretch of amino acid deleted from either the C or N terminus.

The present invention is directed in one aspect to an assay system for a sample suspected of containing hepatitis C viral (HVC) RNA comprising: contacting the sample with an NS3 protein, fragment, or mutant thereof which forms a nucleoprotein complex. In a preferred embodiment, the NS3 comprises a label.

In another aspect the invention is directed to a method for inhibiting the binding of helicase to HCV RNA comprising contacting the HCV RNA with an NS3 protein, fragment or mutant, thereof to form a nucleoprotein complex, wherein the binding of helicase to the HCV RNA is inhibited.

In another aspect, the invention is directed to a method for assaying a sample containing a viral RNA comprising incubating the sample with a protein, fragment or mutant thereof to form a nucleoprotein complex, and quantitating the amount or presence of the viral RNA that binds to the protein.

In another aspect, the invention is directed to a kit for assaying a sample containing a viral RNA wherein a protein, fragment or mutant thereof is capable of forming a nucleoprotein complex with a viral RNA. In a preferred embodiment, the kit also comprises instructions for use.

In yet another aspect, the invention is directed to a method for treating a subject having HCV infection comprising administration of an agent to the subject which inhibits the binding of helicase to HCV RNA. Preferably, the agent is a NS3 protein, fragment or mutant thereof. In another embodiment, the agent is an antibody. In yet another

embodiment, the agent is a small molecule chemical compound. Preferably the subject is human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of HCV genome organization marking the relative position of NS3 protein. The numbers correspond to nucleotide positions in the HCV 1969 cDNA clone.

Figure 2 is a schematic representation of the viral positive-strand UTR used in the study of HCV-encoded NS3-RNA interaction.

Figure 3 is the predicted secondary structure of the first 127 nucleotides of the sequence of the positive strand, 5'(+) UTR_{127} (adapted from reference 20).

Figure 4 is a schematic illustration of the mutant RNAs used in the binding assay of HCV-encoded ΔpNS3 interaction with negative-strand 3' UTR_{127} mutant RNA probes. Mutants with deleted (mutants I, II, and VII) or altered (mutants IV, V, and VI) bases compared to the wild-type RNA are indicated by shading and/or *, respectively. The intervening sequences are indicated by dots.

Figure 5 is a schematic illustration of the mutant RNAs used in the binding analysis of NS3 and ΔpNS3 binding to the 3' negative-strand UTR_{127}. The altered bases in the mutant RNAs compared to the wild-type RNA are shaded and marked by asterisks. The intervening sequences are indicated by dots.

Figure 6 is a schematic illustration of the various mutant probes used for RNA-protein binding analysis. All of the probes contain mutant V RNA as a backbone. The reversion to the wild-type sequence of specific C-GG-C base pairs in the 3'-proximal stem is marked by shading and an asterisk. The intervening sequences are indicated by dots.

Figure 7 is a schematic illustration of the mutant RNA probes used in binding analysis of NS3 and ΔpNS3 protein binding to 3' negative-strand UTR_{127} RNA mutants with a single G-CC-G change. The base pair sequences in the mutant RNAs that are altered compared to the wild-type sequence are marked by shading and asterisks. The intervening sequences are indicated by dots.

Figure 8 is a schematic illustration of the mutant RNA probes used in the binding analysis of NS3 binding to additional 3'UTR_{127} RNA mutants with specific G-CA-U changes. The base pair sequence in the mutant RNAs that have been altered compared to the wild-type sequence are marked by shading and asterisks. The intervening sequences are indicated by dots.

Figure 9 is a schematic representation of the viral 3' UTR RNA and the derived mutants used in analysis of NS3 binding to the 3' UTR of the positive strand. The mutants with deleted RNA sequences (mutants A to D) are shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to binding assay and methods for the measurement of Hepatitis C virus (HCV) helicase and methods of inhibiting the binding of helicase to HCV RNA based on results from the studies of the interaction of HCV encoded NS3 helicase protein with the 3'-terminal sequences of viral positive- and negative-strand RNA.

Definitions

The terms "Hepatitis C Virus" and "HCV" refer to the viral species that is the major etiological agent of BB-NANBH, the prototype isolate of which is identified in WO89/046699; EPO publication 318,216; and. U.S. Pat. No. 5,350,671, the disclosures of which are incorporated herein by reference. "HCV" as used herein includes the pathogenic strains capable of causing hepatitis C, and attenuated strains or defective interfering particles derived therefrom. The HCV genome is comprised of RNA. It is known that RNA-containing viruses have relatively high rates of spontaneous mutation,

reportedly on the order of 10^{-3} to 10^{-4} per incorporated nucleotide (Fields & Knipe, "Fundamental Virology" (1986, Raven Press, N.Y.)). As heterogeneity and fluidity of genotype are inherent characteristics of RNA viruses, there will be multiple strains/isolates, which may be virulent or avirulent, within the HCV species.

The phrases "nucleic acid" or "nucleic acid sequence" as used herein refer to an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin.

A "coding sequence of" or a "nucleotide sequence encoding" a particular polypeptide or protein, is a nucleic acid sequence which is transcribed and translated into a polypeptide or protein when placed under the control of appropriate regulatory sequences.

As used herein, a polynucleotide "derived from" a designated sequence refers to a polynucleotide sequence which is comprised of a sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, and even more preferably at least about 15-20 nucleotides corresponding to a region of the designated nucleotide sequence. "Corresponding" means homologous to or complementary to the designated sequence. Preferably, the sequence of the region from which the polynucleotide is derived is homologous to or complementary to a sequence which is unique to an HCV genome. Whether or not a sequence is unique to the HCV genome can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks, e.g., Genebank, to determine whether it is present in the uninfected host or other organisms. The sequence can also be compared to the known sequences of other viral agents, including those which are known to induce hepatitis, e.g., HAV, HBV, and HDV, and to members of the *Flaviviridae*. The correspondence or non-correspondence of the derived sequence to

other sequences can also be determined by hybridization under the appropriate stringency conditions. Hybridization techniques for determining the complementarity of nucleic acid sequences are known in the art. See also, for example, Maniatis et al. (1982). In addition, mismatches of duplex polynucleotides formed by hybridization can be determined by known techniques, including for example, digestion with a nuclease such as S1 that specifically digests single-stranded areas in duplex polynucleotides. Regions from which typical DNA sequences may be "derived" include but are not limited to, for example, regions encoding specific epitopes, as well as non-transcribed and/or non-translated regions.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence shown, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use.

Similarly, a polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence, for example, the sequences in Section IV.A, or from an HCV genome; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from HCV, including mutated HCV. A recombinant or derived polypeptide may include one or more analogs of amino acids or unnatural amino acids in its sequence. Methods of inserting

analogs of amino acids into a sequence are known in the art. It also may include one or more labels, which are known to those of skill in the art.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

"Amino acid" or "amino acid sequence" as used herein refer to an oligopeptide, peptide, polypeptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring or synthetic molecules.

The term "polypeptide" or "polyprotein" as used herein, refers to amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphytidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristolyation, oxidation, pergylation, proteolytic processing, phosphorylation, prenylation, racemization,

selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. (*See* Creighton, T.E., Proteins – Structure and Molecular Properties 2nd Ed., W.H. Freeman and Company, New York (1993); *Posttranslational Covalent Modification of Proteins*, B.C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983)).

As used herein, the term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

As used herein, the term "recombinant" means that the nucleic acid is adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. Additionally, to be "enriched" the nucleic acids will represent 5% or more of the number of nucleic acid inserts in a population of nucleic acid backbone molecules. Backbone molecules according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. Typically, the enriched nucleic acids represent 15% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. More typically, the enriched nucleic acids represent 50% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. In a one embodiment, the enriched nucleic acids represent 90% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules.

"Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an

exogenous DNA construct encoding the desired polypeptide or protein. "Synthetic" polypeptides or protein are those prepared by chemical synthesis. Solid-phase chemical peptide synthesis methods can also be used to synthesize the polypeptide or fragments of the invention. Such method have been known in the art since the early 1960's (Merrifield, R. B., *J. Am. Chem. Soc.*, 85:2149-2154, 1963) (See also Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, Ill., pp. 11-12)) and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have generally utilized the teachings of H. M. Geysen *et al, Proc. Natl. Acad. Sci., USA*, 81:3998 (1984) and provide for synthesizing peptides upon the tips of a multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, *i.e.*, inverting and inserting the rod's and pin's tips into appropriate solutions, amino acids are built into desired peptides. In addition, a number of available FMOC peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A automated peptide synthesizer. Such equipment provides ready access to the peptides of the invention, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

A promoter sequence is "operably linked to" a coding sequence when RNA polymerase which initiates transcription at the promoter will transcribe the coding sequence into mRNA.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or

more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

The term "purified viral polynucleotide" refers to an HCV genome or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90% of polypeptides with which the viral polynucleotide is naturally associated. Techniques for purifying viral polynucleotides from viral particles are known in the art, and include for example, disruption of the particle with a chaotropic agent, differential extraction and separation of the polynucleotide(s) and polypeptides by ion-exchange chromatography, affinity chromatography, and sedimentation according to density.

The term "purified viral polypeptide" refers to an HCV polypeptide or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90%, of cellular components with which the viral polypeptide is naturally associated. Techniques for purifying viral polypeptides are known in the art.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may

not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, and recombinant polynucleotide sequences.

"Immunologically identifiable with/as" refers to the presence of epitope(s) and polypeptides(s) which are also present in the designated polypeptide(s), usually HCV proteins. Immunological identity may be determined by antibody binding and/or competition in binding; these techniques are known to those of average skill in the art.

As used herein, "epitope" refers to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

A polypeptide is "immunologically reactive" with an antibody when it binds to an antibody due to antibody recognition of a specific epitope contained within the polypeptide. Immunological reactivity may be determined by antibody binding, more particularly by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. The techniques for determining whether a polypeptide is immunologically reactive with an antibody are known in the art.

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one antibody combining site. An "antibody combining site" or "binding domain" is formed from the folding of variable domains of an antibody molecules) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows an immunological reaction with the antigen. An antibody combining site may be formed from a heavy and/or a light chain domain (VH and VL, respectively), which form hypervariable loops which contribute to antigen binding. The term "antibody" includes, for example, vertebrate antibodies, hybrid antibodies, chimeric

antibodies, altered antibodies, univalent antibodies, the Fab proteins, and single domain antibodies.

As used herein, a "single domain antibody" (dAb) is an antibody which is comprised of an VH domain, which reacts immunologically with a designated antigen. A dAB does not contain a VL domain, but may contain other antigen binding domains known to exist in antibodies, for example, the kappa and lambda domains. Methods for preparing dABs are known in the art. See, for example, Ward et al. (1989).

Antibodies may also be comprised of VH and VL domains, as well as other known antigen binding domains. Examples of these types of antibodies and methods for their preparation are known in the art (see, e.g., U.S. Pat. No. 4,816,467, which is incorporated herein by reference), and include the following. For example, "vertebrate antibodies" refers to antibodies which are tetramers or aggregates thereof, comprising light and heavy chains which are usually aggregated in a "Y" configuration and which may or may not have covalent linkages between the chains. In vertebrate antibodies, the amino acid sequences of all the chains of a particular antibody are homologous with the chains found in one antibody produced by the lymphocyte which produces that antibody *in situ*, or *in vitro* (for example, in hybridomas). Vertebrate antibodies typicalily include native antibodies, for example, purified polyclonal antibodies and monoclonal antibodies. Examples of the methods for the preparation of these antibodies are known to those of skill in the art.

"Hybrid antibodies" are antibodies wherein one pair of heavy and light chains is homologous to those in a first antibody, while the other pair of heavy and light chains is homologous to those in a different second antibody. Typically, each of these two pairs will bind different epitopes, particularly on different antigens. This results in the property of "divalence", i.e., the ability to bind two antigens simultaneously. Such hybrids may also be formed using chimeric chains, as set forth below.

"Chimeric antibodies", are antibodies in which the heavy and/or light chains are fusion proteins. Typically the constant domain of the chains is from one particular species and/or class, and the variable domains are from a different species and/or class. Also included is any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, or different species of origin, and whether or not the fusion point is at the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation, or to make other improvements in properties possessed by a particular constant region.

Another example is "altered antibodies", which refers to antibodies in which the naturally occurring amino acid sequence in a vertebrate antibody has been varied. Utilizing recombinant DNA techniques, antibodies can be redesigned to obtain desired characteristics. The possible variations are many, and range from the changing of one or more amino acids to the complete redesign of a region, for example, the constant region. Changes in the constant region, in general, to attain desired cellular process characteristics, e.g., changes in complement fixation, interaction with membranes, and other effector functions. Changes in the variable region may be made to alter antigen binding characeristics. The antibody may also be engineered to aid the specific delivery of a molecule or substance to a specific cell or tissue .site. The desired alterations may be made by known techniques in molecular biology, e.g., recombinant techniques, site directed mutagenesis, etc.

Yet another example are "univalent antibodies", which are aggregates comprised of a heavy chain/light chain dimer bound to the Fc (i.e., constant) region of a second heavy chain. This type of antibody escapes antigenic modulation. See, e.g., Glennie et al. (1982).

Included also within the definition of antibodies are "Fab" fragments of antibodies. The "Fab" region refers to those portions of the heavy and light chains which are roughly equivalent, or analogous, to the sequences which comprise the branch portion of the heavy and light chains, and which have been shown to exhibit immunological binding to a specified antigen, but which lack the effector Fc portion . "Fab" includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers containing the 2H and 2L chains (referred to as F(ab).sub.2), which are capable of selectively reacting with a designated antigen or antigen family. "Fab" antibodies may be divided into subsets analogous to those described above, i.e, "vertebrate Fab", "hybrid Fab", "chimeric Fab", and "altered Fab". Methods of producing "Fab" fragments of antibodies are known within the art and include, for example, proteolysis, and synthesis by recombinant techniques.

As used herein, the term "immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Treatment" as used herein refers to prophylaxis and/or therapy.

An "individual", as used herein, refers to vertebrates, particularly members of the mammalian species, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

As used herein, the "sense strand" of a nucleic acid contains the sequence that has sequence homology to that of mRNA. The "anti-sense strand" contains a sequence which is complementary to that of the "sense strand".

As used herein, a "positive stranded genome" of a virus is one in which the genome, whether RNA or DNA, is single-stranded and which encodes a viral polypeptide(s). Examples of positive stranded RNA viruses include *Togaviridae, Coronaviridae, Retroviridae, Picornaviridae, and Caliciviridae.* Included also, are the *Flaviviridae,* which were formerly classified as *Togaviradae.* See Fields & Knipe (1986).

As used herein, "antibody containing body component" refers to a component of an individual's body which is a source of the antibodies of interest. Antibody containing body components are known in the art, and include but are not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, white blood cells, and myelomas.

As used herein, "purified HCV" refers to a preparation of HCV which has been isolated from the cellular constituents with which the virus is normally associated, and from other types of viruses which may be present in the infected tissue. The techniques for isolating viruses are known to those of skill in the art, and include, for example, centrifugation and affinity chromatography.

The term "HCV particles" as used herein include entire virion as well as particles which are intermediates in virion formation. HCV particles generally have one or ore HCV proteins associated with the HCV nucleic acid.

As used herein, the term "probe" refers to a polynucleotide which forms a hybrid structure with a sequence in a target region, due to complementarity of at least one sequence in the probe with a sequence in the target region.

As used herein, the term "target region" refers to a region of the nucleic acid which is to be amplified and/or detected.

As used herein, the term "viral RNA", which includes HCV RNA, refers to RNA from the viral genome, fragments thereof, transcripts thereof, and mutant sequences derived therefrom.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components).

"Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described herein are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion, gel electrophoresis may be performed to isolate the desired fragment.

"Oligonucleotide" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

The phrase "substantially identical" in the context of two nucleic acids or polypeptides, refers to two or more sequences that have at least 60%, 70%, 80%, and in some aspects 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the known sequence comparison algorithms or by visual inspection. Typically, the substantial identity exists over a region of at least about 100 residues, and most commonly the sequences are substantially identical over at least about 150-200 residues. In some embodiments, the sequences are substantially identical over the entire length of the coding regions.

Additionally a "substantially identical" amino acid sequence is a sequence that differs from a reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site of the molecule, and provided that the polypeptide essentially retains its functional properties. A conservative amino acid substitution, for example, substitutes one amino acid for another of the same class (*e.g.*, substitution of one hydrophobic amino acid, such as isoleucin, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid or glutamine for asparagine).

"Fragments" as used herein are a portion of a naturally occurring protein which can exist in at least two different conformations. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. "Substantially the same" means that an amino acid sequence is largely, but not entirely, the same, but retains at least one functional activity of the sequence to which it is related.

In general two amino acid sequences are "substantially the same" or "substantially homologous" if they are at least about 85% identical. Fragments which have different three dimensional structures as the naturally occurring protein are also included. An example of this, is a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

"Hybridization" refers to the process by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37°C to 42°C. Hybridization could occur under reduced stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization could occur under high stringency conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 n/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

The term "variant" refers to polynucleotides or polypeptides of the invention modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) yet still retain the biological activity of NS3. Variants can be produced by any number of means included methods such as, for example, shuffling, oligonucleotide-

directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, and any combination thereof.

Description

In one embodiment, the present invention provides a method for assaying a sample suspected of containing hepatitis C viral RNA. The assay comprises contacting the sample with an NS3 protein, fragment, or mutant thereof to form a nucleoprotein complex; and measuring the amount or presence of the HCV RNA in the sample. The contact of the sample with an NS3 protein, fragment, or mutant thereof may be for a defined period of time. The NS3 protein may also be a fragment or mutant variety which could be truncated version of the wild type protein (the truncation could be from the N or C terminus) or a mutation confined to a single or double amino acids residue in the NS3 protein. In another aspect the protein could be only the helicase portion of the NS3 protein. The complex formation would be indicative of the presence of the viral RNA in the sample. In a preferred embodiment, the NS3 protein, fragment or mutant thereof comprises a label.

In another aspect, the invention is directed to a kit for assaying a sample suspected of containing a HCV RNA, wherein the kit comprises a composition having an NS3 protein, fragment or mutant thereof. The kit can be used as an assay to measure the presence or amount of HCV RNA. In a preferred embodiment, the NS3 comprises a label. Preferably, the kit contains instructions for performing the assay.

In yet another aspect, the invention is directed to an assay system for a sample suspected of containing hepatitis C viral (HVC) RNA comprising contacting the sample with an NS3 protein, fragment, or mutant thereof which forms a nucleoprotein complex. In a preferred embodiment, the NS3 comprises a label.

In another embodiment the invention provides a method for inhibiting the binding of helicase to HCV RNA, comprising contacting a sample with an NS3 protein, fragment, or mutant thereof to form a nucleoprotein complex, wherein the binding of helicase to the HCV RNA is inhibited.

In another embodiment, the invention is directed to a kit for assaying a sample suspected of containing a viral RNA, where the kit comprises a protein, fragment or mutant thereof that is capable of forming a nucleoprotein in complex with a viral RNA. The kit can be used as an assay to measure the presence or level of viral RNA. In a preferred embodiment, the NS3 comprises a label. Preferably, the kit contains instructions for assaying the sample.

In a preferred embodiment, a kit contains instructions for performing the assay, which instructions may be printed on a package insert, packaging or label included in the kit. The printed matter can also be included on receptacles included in the kit, and indicia of sample and reagent volumes can be indicated in the test receptacle. The precise instructions would vary depending upon the protein or substance to be detected and/or detection method used, but may include instructions for one or more of the following: instructions for dilution of the kit components and/or preparation of the sample, directions for volume or concentration of labeled protein, fragment or mutant thereof used for each assay, volume of sample to add to the assay, directions for labeling the reactants, directions for taking measurement of labeled components, preferred temperature conditions, and timing of component addition and mixing, and use of a standard to calibrate test results.

Preferably in the above mentioned kits or assays, the HCV RNA contains either the 3' positive or the 3' negative strand UTR sequences. More preferably the first 127 bases of the positive strand and the terminal sequences of the negative strand designated as 3'(+) UTR $_{127}$ or 3'(-) UTR $_{127}$. The positive strand UTR$_{127}$ RNA could serve as the negative control. In another embodiment, the RNA contains the stem loop structure of

the negative strand UTR RNA that is found near the 3' terminus of the RNA or the corresponding stem loop structure of the positive strand. Most preferably the HCV RNA comprises nucleotides −7 through −9 of the stem loop structure. The minus sign is indicative of the base numbers in the negative strand of the RNA where the NS3 protein is suspected to bind and is complimentary to the negative strand.

In another aspect, the invention is directed to a method for assaying a sample containing a viral RNA comprising: contacting the sample with a protein, fragment, or mutant of said protein to form a nucleoprotein complex, and measuring the amount or presence of the viral RNA that binds to the protein. Such a binding assay is used to determine whether a similar binding complex, in comparison to the NS3-HCV RNA nucleoprotein complex, is formed. Preferably, the protein is a helicase and more preferably a NS portion of a helicase or other portion that binds to the RNA. Preferably, the RNA is a 3'-terminal sequence of viral positive- or negative-strand RNA of the virus. Preferably the viral RNA is *Flaviviridae* viral RNA and preferably the protein, fragment, or mutant of said protein is a protease and/or helicase protein, fragment, or mutant of said protein.

Other viruses that contain conserved helicase motifs in their NS3 proteins are also candidates for the above-described assays. In addition to flaviviruses, pestiviruses can also be the subject of the binding assay, method or kit. Further, other positive-stranded RNA viruses, such as poliovirus, rhinovirus, and coxsackie virus, also encode a distinct but homologous protein (2C) with RNA binding and NTPase activities and putative helicase activity and thus can also be candidates for the binding assay, method or kit.

Mutations of the RNA can be performed to determine the important binding sites of the RNA, as was similarly performed for the HCV RNA.

A sample preferably comprises any fluid containing the HCV RNA which is often a preparation or fraction of a biological sample. Preferably a biological sample is derived from a sample of blood, liver cells, or other extract taken from a mammal, preferably a

human, to be tested for the presence of RNA. Preferable the RNA in the sample is isolated. More preferably the sample is a preparation of liver cells or whole blood.

An assay of the invention can include a heterogeneous or homogenous assay and includes any suitable methodology used for antibodies. "Homogeneous assay" refers to an assay in which the presence and/or concentration of a protein is determined without requiring the separation of sample fluid from the reaction components. A homogenous assay includes formats in which a detectable signal is only generated upon specific binding of a labeled protein to the RNA. As such, homogenous assay formats, the detection occurs without a non-bound labeled protein removal step. This broad classification includes many formats known to those skilled in the art.

"Heterogeneous assay" refers to an assay in which a complex is formed, which is removed from the reaction medium before measuring.

In a preferred embodiment of the method or assay, the protein comprises a label. The label can be an intrinsic or extrinsic label. More preferably, the label is a fluorescent label, a luminescent label, an enzyme label, a radioactive label, or a chemical label.

It is shown in the present invention that the HCV-encoded protease/helicase protein NS3 interacts with the 3'-terminal sequences of the viral positive-and negative-strand RNA. Specifically, the helicase portion of NS3 is responsible for RNA-protein interaction, as indicated by the similar binding profiles of the full-length NS3 protein and the truncated protein lacking the protease domain (ΔpNS3). The interactions of NS3 with the 3' UTR_{127} of the negative-strand RNA and the 3' UTR of the positive-strand RNA appear to be specific, since the formation of the nucleoprotein complexes is successfully competed by the unlabeled homologous RNA but not by similar-size RNAs derived from hepatitis A virus or poliovirus. The specificity of NS3-RNA interaction is also validated by the fact that only the 3'(-) UTR_{127} and 3'(+) UTR sequences, but not the corresponding complementary sequences from the 5'(+) and 5'(-) termini, interact with NS3. The interaction of NS3 with the 3'-terminal sequences of the negative-strand UTR RNA

appears to depend on a stem-loop structure near the 3' terminus of the RNA. Additional mutagenesis confined to the stem-loop structure spanning nucleotides 5 through 20 suggests that an intact double-stranded structure of the stem and the orientation of three G-C pairs (at positions -7, -8, and -9) within this stem are important elements for specific interaction of NS3 with the negative-strand RNA.

In the past, various investigators have reported on the RNA binding activity of the helicase portion of NS3. The majority of the RNA binding studies reported to date have used partially single-stranded or single-stranded RNA, including homopolymeric RNAs. Kanai et al. demonstrated binding of NS3 to poly(U) Sepharose resin with an apparent dissociation constant of 2 × 107 M (26). Other investigators have used filter binding (35), gel retardation (19), and fluorescence quenching (43) assays to assess the RNA binding activity of NS3. Except for some preference for poly(U), none of these studies detected any specific interaction of NS3 with HCV RNA. The preference for poly(U) can be explained by the fact that poly(U) stretches are present at the 3' UTR terminus in HCV positive-strand RNA. The binding results of the present invention, along with those of the competition analysis, clearly demonstrate that the requirements of NS3 interaction with the 3'(+) UTR are much more complex than the mere presence of the poly(U) stretch. In fact, the 5' half of the 3'(+) UTR, which contains the poly(U) stretch, is unable to interact with NS3 (or ΔpNS3) in the absence of the 3' half of the UTR under the assay conditions. In contrast to the relatively stringent requirement for 3'(-) UTR_{127} binding, the interaction of NS3 with the 3'(+) UTR appears to require an intact structure of the entire 3'(+) UTR RNA under the assay conditions used in this study. The present invention is the first demonstration of a specific interaction of HCV NS3 protein with the 3'-terminal sequences of the HCV RNA. The importance of the 5' and 3' UTR sequences in viral infectivity have recently been confirmed in the chimpanzee model (32, 56-58). It should be pointed out that the 3'-terminal sequences of the negative- and positive-strand UTR contain some extra nucleotides that are contributed by the multiple cloning site. All pestiviruses and flaviviruses contain conserved helicase motifs in their NS3 proteins, suggesting an important role of the helicase in the life cycles of these viruses. Other

positive-strand RNA viruses, such as poliovirus, rhinovirus, and coxsackie virus, also encode a distinct but homologous protein (called 2C) with RNA binding and NTPase activities and putative helicase activity (39, 41). It has previously been shown that the poliovirus-encoded 2C protein specifically interacts with the 3' UTR sequences of the viral negative-strand RNA (2). Both the precursor, 2BC, and the mature protein, 2C, are also capable of interacting with the cellular membranes (5, 10, 13, 14). It has been postulated that 2C may anchor viral negative-strand RNA to the cytoplasmic membrane so that initiation of positive-strand RNA synthesis can occur at the 3' end of negative-strand RNA (2). Given the functional similarity between poliovirus 2C and the HCV helicase (NS3 and ΔpNS3), NS3 may have a similar role in HCV RNA synthesis. Recent results have suggested that interaction of NS3 with NS4A directs NS3 to the endoplasmic reticulum (54). While not wanting to be bound by a particular theory, since NS3 appears to interact with the 3' UTR of the positive- as well as the negative-strand RNA, a likely scenario might be that the NS3-NS4A complex bound to either the positive- or negative-strand 3' UTR may anchor RNA-protein complexes to the cytoplasmic membrane. The polymerase NS5B (and possibly NS5A) can then join this complex by protein-protein and/or protein-RNA interaction and initiate positive- or negative-strand RNA synthesis. In fact, direct interaction of HCV NS5B with NS3 and NS4A has been reported (23). Also, in the closely related dengue virus, another member of the family *Flaviviridae*, both NS3 and NS5 have been shown to interact *in vivo* in CV-1 and HeLa cells (27). One or more host cell proteins could play a role in the membrane association of the viral RNA replication complex through participation of a membrane-associated protein, hVAP-33 (52). This SNARE-like protein containing a membrane-spanning domain has been shown to interact with both NS5A and NS5B. As reliable systems to study HCV RNA replication become available, it will be possible to address mechanistic questions to assess the roles of various viral proteins in RNA replication. The results presented here do not exclude the possibility that NS3 also interacts with other regions of viral positive- or negative-strand RNA. However, since initiation of RNA synthesis is likely to start at the 3' terminus of positive- and negative-strand RNA, it is likely that the interaction

presented here is important for viral RNA replication. It is also important to note that specific binding of a protein to an RNA sequence and/or structure does not necessarily provide functional relevance to the RNA-protein interaction. Additional studies must be conducted to assess the importance of the mutations in both the 3'(-) and 3'(+) UTR that alter NS3 binding. The present invention demonstrates specific interaction of the HCV NS3 (helicase domain) with the 3'(-) and 3'(+) UTR sequences. The initial mutagenesis studies reported here have confirmed the requirement for the specific structure and sequence in NS3 binding. The binding of NS3 to the 3'-terminal sequences of positive- and negative-strand RNA plays an important role in HCV RNA replication.

The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

EXAMPLES

Example 1: Expression and purification of HCV NS3 & ΔpNS3

The recombinant clone used for expression of the viral wild type NS3 and the truncated version, ΔpNS3 [with an intact helicase but deleted protease domain] was constructed by PCR amplification using the HCV 1969-cDNA clone (a kind gift from Dr. Genevieve Inchauspe, INSERM, France)as the template. The coding sequence was aligned with past published data and primers were designed for both the proteins as follows:

forward (RB-1) 5'-ATATTAAGGATCCGGCGCCCATCACGG-3' for NS3 and forward primer (RB-3) 5'-CGCAATAGGATCCGGTGGACTTTATCCC-3' for ΔpNS3 and

reverse primer (RB-2) 3'-ATTAATTAAGCTTCGTGACAACCTCCAGGT-5' that was used for cloning both genes.

Each oligonucleotide had either a Bam H-I or Hind III restriction site incorporated in it (underlined) and an additional G base following it to preserve the frame. The

amplified, gel-purified, sequentially digested PCR product was ligated into the corresponding sites of vector plasmid pET 21b (Novagen) using T4 ligase as per standard protocol. Familiar in the art to a skill worker *E.coli* SCS I cells were transformed and stable transformants were selected for ampicillin resistance. The resulting plasmid encoding either full length or truncated NS3 contained an eleven amino acid T7 tag at the N-terminus for detection in Westerns, and a six histidine sequence at the C-terminus for ease of purification using metal-affinity chromatography. The nucleotide sequences of the recombinant clones were confirmed by restriction digestion as well as by dideoxy-sequencing. These clones were used throughout this study, for *in vitro* transcription-translation and also transformed into *E.coli* strain, BL21(DE3), for expression and purification of the recombinant protein.

Overnight cultures of *E.coli* BL21(DE3) cells harboring the recombinant plasmids were inoculated into fresh LB media containing ampicillin (100μg/ml). Expression of the target protein was induced by addition of IPTG once the required OD was reached. Cells were harvested 3.5 hours post-induction and the expressed proteins were purified from lysate using Co^{++} charged resin [Talon, Clonetech] as detailed in reference (1). The purified proteins were aliquoted into small portions and stored at -80°C to prevent repeated freeze-thawing of the samples. Purity of the isolated protein samples were evaluated by resolving on SDS-polyacrylamide gels followed by Coomassie blue staining. Identity of the isolated NS3 is confirmed by immunoblot analysis (Western) using alkaline phosphatase conjugated secondary antibody and visualized by the chromogenic substrate BCIP-NBT as detailed in reference (1). Alternatively, chemiluminescence could also be employed.

Example 2: UTR cloning and probe preparation

The viral 5'UTR sequence, encompassing first 127 nucleotides was PCR amplified from HCV 1969 cDNA clone using the forward primer (RB-4)

5'- ATATTAGAATTCGCCAGCCCCCTG-3' and reverse primer (RB-5)
3'-TTATATAAGCTTGGGGGGGTCCTGGAG-5'.

These and any other primers sequence could be generated known in the art by a skilled personal. These primers should have appropriate restriction sites incorporated in them (as underlined), The resulting PCR product is gel purified, sequentially digested with Hind III and EcoRI and ligated into the corresponding sites of the transcription vector pGEM-3 (Promega). *E.coli* SCS I (Stratagene) or DH5α (Gibco) cells were transformed with the ligated plasmid and positive transformants were selected. Nucleotide sequence of the recombinant clones (wild type and the mutant UTR's) was confirmed by restriction digestion and dideoxy-sequencing (Amersham). The cloning strategy was chosen such that positive or the complementary negative strand transcripts [5' (+)UTR or 3'(-)UTR]could be generated by *in vitro* transcription reaction using either SP6 or T7 RNA polymerase in the presence of [α^{32}P]-UTP (3000Ci/m mole, Amersham). In order to isolate intact, full length probe, the uniformly labeled [^{32}P]-UTP incorporated RNA transcript was resolved on a denaturing sequencing gel [8% acrylamide: 8M urea], the appropriate band was excised from the wet gel, eluted and subsequently alcohol precipitated. Filter binding assay was used to quantify counts incorporated and probes were aliquoted and stored at –80°C in small fractions for single time use. Prior to using them in binding analysis the probes were uniformly diluted to approximately 100,000 cpm / μl with nuclease-free water and the counts were requantified using the filter binding assay.

The 5' UTR mutant series (I-XXI) were cloned into the same vector following similar PCR strategy as above for cloning the wild type 5' UTR_{127}. The 3' negative strand RNA probes were obtained by transcription off the SP6 promoter sequence in the presence of labeled UTP, followed by purification and elution of the intact RNA probe. The mutants were generated using the forward primer sequence as given while using the same reverse primer sequence as was used in the construction of the wild type 5' UTR_{127} (RB-5), with the exception of mutant VII. For the rescue experiment using mutants V[A]

–[F] as described in figure 6, the PCR fragment was generated with mutant V DNA as template. The small case italics in the oligonucleotides denotes the specific altered bases in the mutants. The forward primer sequences were as follows:

5'-ATCCTCGAATTCTGATGGGGGACA-3'(mutant I),

5'GCATTAGAATTCCACCATGAATCA-3'(mutant II),

5'-AATTAAGAATTCGCCAG*ggggg*TGATGGG-3'(mutant IV),

5'-AATTATGAATTCGCCAGgggggTGAT*ccccc*CGACACTC-3'(mutant V),

5'-TTAAATGAATTCGCCAG*ttttt*TGAT*aaaaa*CGACACTC-3'(mutant VI),

5'-ATATAAGAATTCGCCAG*ttttt*TGATGGGG-3'(mutant VIII),

5'-ATATAAGAATTCGCCAGCCCCCTGAT*aaaaa*CGACACTCCA-3'(mutant IX),

5'-TTAATAGAATTCGCCAGC*g*CCCTGATGGG*c*GCGACACTC-3' (mutant X),

5'-TTAATAGAATTCGCCAGCC*g*CCTGATGG*c*GGCGACACTC-3' (mutant XI),

5'-TTAATAGAATTCGCCAGCCC*g*CTGATG*c*GGGCGACACTC-3' (mutant XII),

5'-ATATAAGAATTCGCCAG*ccc*GGTGATCC*ggg*CGACACTCC-3' (mutant V-[A]), 5'-ATATAAGAATTCGCCAGG*ccc*GTGATC*ggg*CCGACACTCC-3' (mutant V-[B]),

5'-ATATAAGAATTCGCCAGGG*ccc*TGAT*ggg*CCCGACACTCC-3' (mutant V-[C]), 5'-TTAATAGAATTCGCCAGG*c*GGGTGATCCC*g*CCGACACTCC-3'(mutant V-[D]),

5'-TTAATAGAATTCGCCAGG*cc*GGTGATCC*gg*CCGACACTCC-3'(mutant V-[E]),

5'-TTAATAGAATTCGCCAGG*c*G*c*GTGATC*g*C*g*CCGACACTCC-3' (mutant V-[F]),

5'-ATTTATGAATTCGCCAGC*t*CCCTGATGGG*a*GCGACACTC-3' (mutant XIX),

5'-GTCTATGAATTCGCCAGC*tt*CCTGATGG*aa*GCGACACTC-3'(mutant XX),

5'-GTCTATGAATTCGCCAGC*t*C*t*CTGATG*a*G*a*GCGACACTC-3' (mutant XXI)

For mutant VII, the forward primer used was same as for wild type (RB-4) and the reverse primer sequence was

3'-GCAACGAAGCTTCTCATACTAACGCCATGG-5'

The 3'-UTR sequence was PCR amplified from the plasmid pCV- H77C (a kind gift from Dr.Jens Bukh, NIH) containing the infectious full length clone of strain H77. The PCR product was gel purified and cloned into the vector used earlier (pGEM-3) between the Hind III and Eco R-I restriction sites using the forward primer (RB-6)

5'-GCATATGAATTCGATGAACGGGGAGC-3' and reverse primer (RB-7)

3'-ATGGGCAAGCTTACATGATCTGCAGA-5'. The mutant UTR with deleted 98 nucleotide conserved sequence (mutant A) was cloned using RB-6 as forward primer and reverse primer (RB-8) sequence as follows:

3'-CGCGCCAAGCTTATTAAAGAAGGGAAAAAGAAAGG-5'. Both probes were generated using the appropriate polymerase in the transcription reaction and added to binding reactions.

The additional HCV 3'-UTR clone with 13 (U) residues, pHCV-3'(+) and the plasmids containing the 98 nucleotide fragment of the 3' UTR conserved region (mutant B) were kind gifts from Prof. M.Lai, USC. Both these plasmids were digested with Eco RI and the required fragments were gel purified, isolated and the probes were generated using T7 RNA polymerase as in earlier preparations. The mutant RNAs with deleted SL-I (mutant C) and SL-I and II (mutant D) sequence were obtained using pHCV-3'(+) DNA as the template following two cycles of PCR amplification as described earlier (27, 45). In the initial round the DNA sequence was amplified using forward primer 5'-AGACCCAAGCTTAACGGGG-3' and the reverse primer

3'-ACATGATCTGCAGAGAGGCC-5'. The amplified product obtained (without the T7 promoter sequence) was checked, eluted from agarose gel and was used as template for the second cycle using the forward primer,

5'-*TAATACGACTCACTATA*AGACCCAAGCTTAACGGGGAGC-3' and reverse primer 3'-GGCTCACGGACCTTTCACAGCTA-5' to obtain mutant C. Mutant D was obtained using the same forward primer as mutant C but the reverse primer sequence was

3'-CTAGGGCTAAGATGGAGCCACCA-5'. The bold letters in forward primer represent the T7 polymerase promoter sequence fused to the primer. The agarose gel purified PCR fragment was directly used as template in transcription reaction for mutant C and D probe preparation followed by purification before adding to binding reaction. The control probe used in the experiments shown in fig. 9 was generated using T7 RNA polymerase from the plasmid pGEM-4Z with the 5'-UTR sequence (127 bases) ligated in between the Hind III and Eco RI sites. This manipulation was essential in order to use T7 RNA polymerase for 3' (-) UTR_{127} probe synthesis.

Example 3: RNA binding, UV cross linking analysis and probe stability analysis

The reaction (25 μL) contained binding buffer (5mM HEPES [pH 7.9], 25 mM KCl, 2 mM $MgCl_2$, 0.5% glycerol), 20 mM DTT, 2 mM ATP, 15 μg yeast t-RNA, RNasin (30 units) and approximately 100ng of purified protein. The binding reaction was preincubated with the purified protein, NS3 or ΔpNS3 in a 30°C water bath for 5 minutes, following which the [^{32}P]RNA probe (200K cpm / 25μL sample) was added. The incubation was continued for an additional 15 minutes to allow the probe RNA to bind to the protein. At termination, samples were crosslinked by UV irradiation. Excess RNA probe was digested with a cocktail of RNase A and T1 at 37°C for 45 minutes. Samples (30μl) were mixed with an equal volume of 2X SDS sample buffer and resolved after

loading onto 14 % SDS-PAGE gels, processed and autoradiographed. The signal intensity obtained in binding analysis using mutant probes and during competition studies were quantitated using laser densitometer scanner (Molecular Dynamics) and data analyzed using the Image-QuaNT software programe.

In competition assays varying amounts of the cold specific and non specific RNA was preincubated with the protein for 10 minutes at 30°C. Thereafter the RNA probe was added as described earlier and incubation continued for additional 15 minutes in the presence of hot and cold RNAs and samples processed. Integrity of the cold RNAs used were checked before adding to reactions.

To study the effect of various binding buffer composition on the interaction between ΔpNS3 and the 3' (-) UTR the following buffers were used; binding buffer I: 5mM HEPES [pH 7.9], 0.5mM $MgCl_2$, 25 mM KCl, 0.5% glycerol ; binding buffer II: 5mM HEPES [pH 7.9], 2mM $MgCl_2$, 25 mM KCl, 10mM NaCl, 0.5% glycerol and binding buffer III: 5mM HEPES [pH 7.9], 2mM $MgCl_2$, 25 mM KCl, 0.5% glycerol. The binding buffer III was used throughout in this study.

The stability of the [^{32}P] labeled wild type or mutant RNA probes in the presence or absence of the viral proteins was examined following incubation under standard binding condition as detailed above. The samples were deproteinized twice followed by alcohol precipitation of the RNA. Finally, the recovered RNA was suspended in nuclease free water, mixed with RNA loading dye and resolved on a denaturing sequencing gel. After completion of the run, the gel was fixed using 10 % acetic acid – methanol solution, dried and autoradiographed.

Example 4: In-vitro transcription and translation

Standard protocol for *in-vitro* transcription was followed. Briefly the overexpression (recombinant) plasmid clones, pET-NS3 or pET-ΔpNS3 were linearized using Hind III enzyme, gel-purified and used as template for synthesis of mRNA. The

mRNA obtained was used to direct an *in-vitro* translation reaction using rabbit reticulocyte lysate (Promega) according to manufacturer's instruction. *In-vitro* synthesized proteins were labeled using 40 μCi of [^{35}S] methionine (specific activity>100 Ci/mmole; Amersham) during translation reaction and resolved along with UV crosslinked protein samples on SDS-PAGE gels. In selected experiments, the coupled *in-vitro* transcription-translation system (Promega) was used according to manufacturer's instructions.

Example 5: Immunoprecipitation

The *in vitro* translated and [^{35}S] labeled ΔpNS3 or the protein UV crosslinked to [^{32}P] labeled RNA in the protein-nucleotidyl complexes were immunoprecipitated overnight on ice using either monoclonal antibody to NS3 (AUSTRAL Biologicals, CA) or a non-specific antibody targeted towards the mammalian transcription factor, CREB . The protein concentration of the non-specific antibody was determined and used at the same concentration as specific antibody. Three separate UV crosslinked samples were pooled for immunoprecipitations. Immunoprecipitation reactions were incubated at 4°C for 3 hours using 1X RIPA buffer (20 mM Tris-HCl pH 7.5, 0.5% deoxycholate,1% NP-40 and 150 mM NaCl), 0.5mM PMSF and 5 mg/mL BSA. Immune complexes were adsorbed on protein-A sepharose beads (5mg/reaction, Pharmacia) for an hour at 4°C following which beads were washed five times with RIPA buffer to reduce background binding, and washed once with PBS. Proteins were finally eluted using Laemelli sample buffer, resolved on a 14% SDS-PAGE gel followed by fixing and autoradiography.

Example 6: HCV protease/helicase NS3 interaction with the 3' terminus of HCV negative-strand RNA

HCV protease/helicase NS3 interacts specifically with the 3' terminus of HCV negative-strand RNA. In order to examine the interaction of NS3 with the 3'-terminal sequence(s) of HCV negative-strand RNA, both NS3 and the truncated protein (ΔpNS3) were expressed in *E. coli* BL21(DE3) cells. Both proteins contained a T7 tag at the N

terminus and six histidine residues in tandem at the C terminus. The additional amino acids contributed by the epitopes increase the mass of the expressed proteins by approximately 2 kDa. The bacterially expressed proteins were purified by Co^{2+} immobilized affinity chromatography, and the purified polypeptides were analyzed by SDS-PAGE followed by Coomassie blue staining. As shown by a Coomassie blue-stained gel of NS3 and ΔpNS3, both NS3 and ΔpNS3, with estimated molecular masses of 75.6 and 55.6 kDa, respectively, were purified to near homogeneity (approximately 98%). To reconfirm that the proteins seen in the Coomassie blue-stained gel were indeed NS3 and ΔpNS3, Western blot analysis was performed with available antibodies against the N-terminal T7 tag. The signals obtained in the immunoblot analysis corresponded correctly with the expected migrations of the polypeptides.

To determine whether NS3 and ΔpNS3 interact with the terminal sequence of the HCV RNA, ^{32}P- labeled RNA probes representing the first 127 nucleotides from the 5' terminus of positive-strand RNA or the corresponding sequence from the 3' terminus of negative-strand RNA were prepared (Fig. 2). Initial experiments with the full-length 3'(-) UTR showed interaction with NS3. Later, however, it was found that the majority of the NS3 binding was localized within the first 127 nucleotides of the 3'(-) UTR (data not shown). The predicted secondary structure of the 5'(+) UTR_{127} is shown in Fig. 3. The structure of the 5'(+) UTR_{127}, but not that of the 3'(-) UTR_{127}, has been confirmed by chemical and enzymatic analysis (7, 20). The M-fold analysis of the structure of 3'(-) UTR_{127} revealed multiple forms having very similar ΔG values(50 to 48 kcal/mol [data not shown]). A predicted structure of 3'(-) UTR_{127} has been used which resembles that of the 5'(+) UTR_{127} until the actual secondary structure of the 3'(-) UTR_{127} is determined experimentally. The ^{32}P- labeled RNA probes were incubated with purified NS3 and ΔpNS3, and the UV-cross-linked RNA-protein complexes were visualized by SDS-PAGE followed by autoradiography. The results clearly show that both NS3 and ΔpNS3 are capable of interacting with the 3'(-) UTR_{127}, but no detectable complex was seen with the corresponding complementary sequence of the 5'(+) UTR_{127} of HCV RNA in a gel analysis of the purified ΔpNS3 and NS3 binding to the 5' positive- or 3' negative-strand

UTR_{127} RNA probe. No complexes were observed when the NS3 or ΔpNS3 protein was excluded from the complete reaction. The protein-nucleotide complexes migrated slightly more slowly than the [35S] methionine-labeled *in vitro*-translated protein. It has previously been shown that the poliovirus-encoded 2C protein (also with helicase and ATPase activities) migrates more slowly than the protein itself when cross-linked to one or more nucleotides (2). This is presumably due to the negative charges contributed by the nucleotides covalently linked to the protein. The interaction of ΔpNS3 with the 3'(-) UTR_{127} was linear with increasing concentration of the purified protein, and significant binding was obtained when the reaction buffer contained 5 mM HEPES, 2 mM $MgCl_2$, 25 mM KCl, 10 mM NaCl, and 0.5% glycerol. Reducing $MgCl_2$ to 0.5 mM resulted in significant loss of binding. Omitting NaCl from the reaction mixture did not have a significant effect on binding of ΔpNS3 to the 3'(-) UTR_{127}. The formation of the ΔpNS3-RNA complex was sensitive to aurine tricarboxylic acid (ATA), a well-known inhibitor of protein-nucleic acid interaction. Almost total inhibition of binding was observed at 10 μM ATA. Both SDS and proteinase K also inhibited complex formation (data not shown). The extents of nucleoprotein complex formation were similar when ΔpNS3 was preincubated at 30 and 40°C before being added to the binding reaction mixture. However, binding was significantly inhibited when preincubation was performed at 65 and 90°C, indicating the heat-labile nature of the protein.

To rule out the possibility that the RNA-protein complex detected by UV-cross-linking analysis is due to the presence of one or more contaminating *E. coli* proteins, the ΔpNS3-3'(-) UTR complex was immunoprecipitated with an antiserum specific to NS3. As expected, the ΔpNS3-RNA complex was specifically immunoprecipitated by anti-NS3 but not by an unrelated antibody. These results confirmed that the protein-nucleotidyl complex contains ΔpNS3. The same experiment was repeated with purified recombinant NS3, and the results were similar to those described above. The possibility that the T7 and/or His tags fused to the NS3 (or ΔpNS3) protein contributed to RNA binding was ruled out by the demonstration that a mutant NS3 protein that had internal

deletions but still retained the tag was unable to interact with the UTR sequence (data not shown).

Example 7: Specificity of RNA-protein interaction

To determine if the binding of ΔpNS3 to the 3'(-) UTR_{127} RNA was specific, competition binding assays were performed in which unlabeled homologous and heterologous RNAs were added to the binding reaction during formation of the ^{32}P-labeled UTR-ΔpNS3 complex. While the addition of 20- and 50-fold molar excesses of unlabeled 3'(-) UTR_{127} resulted in approximately 60 to 70% reduction in binding, the intensity of the labeled complex was reduced to almost 10% by the addition of a 100-fold molar excess of unlabeled homologous RNA. No significant reduction in the intensity of the RNA-protein complex was noted in the presence of 20-, 50-, and 100-fold molar excesses of cold, heterologous, similar-size RNA from hepatitis A virus. Additionally, when an unrelated similar-size labeled RNA fragment from the HCV negative strand was used in the binding reaction, only approximately 4% as much RNA-ΔpNS3 complex formation was observed as with the wild type 3'(-) UTR_{127} probe. Finally, two mutant 3'(-) UTR_{127} RNAs that were defective in binding NS3 and NS3 (mutants V and VI; see below) were unable to compete with 3'(-) UTR_{127} probe in the binding assay over a range of concentrations. Taken together, these results suggest that the interaction of ΔpNS3 with the 3'(-) UTR_{127} sequence is specific.

Example 8: 3'-Terminal sequences of the negative-strand UTR_{127} RNA are important for protein binding

To determine sequences within the 3' UTR RNA (127 nucleotides) of HCV required for NS3 binding, 10 and 25 nucleotides were initially deleted from the 3' terminus. These mutants were termed Δ10 (mutant I) and Δ25 (mutant II). Compared to the wild-type 3'(-) UTR_{127}, both mutants I and II were defective in RNA binding. While deletion of the first 10 nucleotides from the 3' terminus of the negative-strand (-10; mutant I) (the minus signs preceding the nucleotide numbers denote the position in the negative-strand RNA starting from the 3' end) resulted in almost 80% decrease in RNA

binding, removal of the first 25 nucleotides (-25; mutant II) reduced binding by approximately 85%. In both mutants I and II, a stem-loop structure within the first 20 nucleotides from the 3' end was deleted (Fig. 4). Therefore, the initial results suggested that the predicted stem-loop spanning nucleotides 5 through 20 may be important for ΔpNS3-RNA interaction. To determine if the stem-loop was somehow involved in NS3 binding, the stem was destabilized by replacing the five guanosine residues with cytosines (Fig. 4, mutant IV). As expected, like mutants I and II, mutant IV was also defective in ΔpNS3 binding (18% binding remaining). It therefore appeared that the stem within the stem-loop structure adjacent to the 3' terminus of the negative-strand UTR_{127} RNA was important for NS3 binding. Deletion of the last 27 nucleotides from the UTR RNA (nucleotides 101 through 127; mutant VII) did not significantly alter protein binding (mutant VII). Similar results were obtained when ΔpNS3 was replaced by NS3 in the binding reaction with the above-mentioned mutants (data not shown).

To rule out the possibility that these mutant RNAs were unstable under the binding assay conditions, parallel reactions were examined for RNA stability. After incubation of various mutant ^{32}P-labeled RNA probes with (or without) protein, the labeled RNAs were isolated by phenol-chloroform extraction and examined by gel analysis. As shown by gels, all of the mutant RNAs tested were just as stable as the wild-type 3'(-) UTR_{127} RNA probe.

To address the question of whether the stem-loop structure alone is important for binding or whether the identities of the bases within this structure are also relevant, the five guanosine and cytosine pairs were replaced by the alternative purine and pyrimidine bases, adenosine and uridine (mutant VI). In this case, no significant binding of ΔpNS3 (or NS3 [data not shown]) was observed. This suggested that the presence of guanosine-cytosine pairs in the stem rather than just a double-stranded structure was important for interaction with NS3. Also, when the positions of the five consecutive guanosine-cytosine pairs in wild-type RNA were flipped (nucleotides 6 through 10; mutant V), no protein-RNA complex could be detected. Again, as shown in gels performed, this was not

due to reduced stability of the mutant RNAs. The relative importance of the five guanosine residues from positions 6 through 10 for protein interaction was also demonstrated by replacing either the five guanosines or the five cytosines in the stem with adenosines and uridines, respectively (Fig. 5). While G-to-A substitutions reduced ΔpNS3 binding by 86% over the control (mutant VIII), C-to-U substitutions reduced binding by only 32% (mutant IX). When NS3 was used in the binding assay, the results were more pronounced; while G-to-A substitution affected binding by 95%, C-to-U changes inhibited binding by only 20% of the control level. To test the possibility that an intact double-stranded structure of the stem is important for RNA-protein interaction, the stem was destabilized by replacing all cytosine residues with guanosine residues (mutant IX-A) (Fig. 5). This mutant affected ΔpNS3 and NS3 binding by 92 and 85%, respectively, compared to the wild-type control, suggesting the importance of an intact helical structure of the stem. To test the contribution of the ACUA loop in protein binding, these nucleotides were replaced by GCCG (mutant IX-B) (Fig. 5). These changes in the loop did not have any significant effect on NS3 (or ΔpNS3) binding. These results indicated that in addition to the double-stranded structure, the presence of the five guanosines in the 3'-proximal arm of the stem was important for interaction with NS3.

Example 9: G-C pairs at positions -7, -8, and -9 in the 3' UTR_{127} RNA-proximal stem are important for protein binding

To determine if all five G-C pairs present in the stem were important for NS3 binding, mutations were introduced into the binding-inactive backbone of mutant V to restore NS3 binding. It is important to note that in mutant V RNA, the positions of the five Gs and Cs in the stem were flipped so that the Cs were 3' proximal in the mutant instead of the Gs being 3' proximal, as in the wild-type 3'(-) UTR_{127}. Replacing three C-G pairs at positions -6, -7, and -8 in the mutant V backbone with the wild-type G-C sequence (mutant V-[A]) restored only 26% binding compared to the wild-type while having three G-C pairs at positions -8, -9, and -10 (mutant V-[C]) did not bring back any significant level of ΔpNS3 binding. Similar results were obtained when ΔpNS3 was

replaced with NS3 in the binding reaction. Restoring three G-C base pairs at positions -7, -8, and -9 (mutant V-[B]), however, brought back 60 to 85% of the binding activity compared to the wild type. Mutant V-[D], in which only the -7 position had a G-C reversion, retained only 2% of the binding activity, and introducing two G-C base pairs at positions -7 and -8 brought the binding activity to only 8% of the control. Interestingly, replacing two G-C pairs at positions -7 and -9, however, restored 50 to 70% of the binding activity compared to that of the wild-type UTR_{127} RNA (mutant V-[F]). These results suggested that the G-C base pairs at positions -7, -8, and -9 in the negative strand may be important for protein binding.

To confirm the above results, specific point mutations confined within these G-C base pairs were introduced into the wild-type UTR_{127} RNA backbone. Changing the G-C pair at position -7 to the flipped sequence C-G (mutant X) severely impaired both ΔpNS3 and NS3 binding compared to the wild-type (2% of the binding remained). Mutant XI, with the G-C at position -8 changed to C-G, retained approximately 20 to 35% of the binding, while changing the G-C base pair at position -9 to C-G in mutant XII had only a marginal effect, if any. These results suggested that the G-C pairs in the stem at the -7 and -8 positions are critical for interaction of 3'(-) UTR_{127} with NS3.

Consistent with the results obtained with NS3 and mutant V and VI UTR protein interaction, the G-C base pair was replaced with the purine-pyrimidine pair, A-U, either singly or in double pairs at the -7, -8, and -9 positions in the wild-type RNA backbone (Fig. 8). As anticipated, significant loss of binding (85% compared to the wild-type control RNA) was apparent when the base pairs at positions -7 and -8 (mutant XX) were replaced. However, by contrast, 71 to 83% of the binding (compared to the wild-type RNA) remained when either the base pair at position -7 (mutant XIX) or the base pairs at positions -7 and -9 (mutant XXI) were changed to A-U. It is worth pointing out that while replacement of the G-C pair at the 7 position in the wild-type backbone with a C-G pair decreased protein-RNA interaction drastically, the replacement of the same G-C pair with A-U did not significantly alter NS3-RNA interaction. The precise reason for this

discrepancy is not clear, but it could be due to the fact that the presence of a purine base at position -7 of the 3'-proximal stem may be crucial for specific binding.

Example 10: NS3 also interacts with the 3'-terminal sequences of the positive-strand RNA.

Since initiation of negative-strand synthesis is likely to occur at or near the 3' terminus of the positive-strand RNA, it was examined whether NS3 is capable of interacting with the 3'-terminal sequences of the positive-strand RNA. The 3' UTR sequence of genomic RNA was cloned into the transcription vector, and RNA was transcribed from the clone, similar to the procedure with the negative-strand UTR_{127} probe. The ^{32}P-labeled 3'(+) UTR RNA was incubated with purified recombinant NS3, and the resulting RNA-protein complexes were analyzed by UV cross-linking. NS3 was seen to readily form complexes with the 3'(+) UTR of HCV. The intensity of the protein-nucleotidyl complex was 70% of that with 3'(-) UTR_{127} RNA. The 5'-terminal sequences of the negative strand (5'(-) UTR) that are complementary to the 3'(+) UTR sequence showed very little binding to NS3 (approximately 3% compared to the 3'(+) UTR binding). These results suggested that NS3 is capable of interacting with the 3'-terminal sequences of both positive- and negative-strand RNA.

The 3'(+) UTR of HCV RNA contains a variable sequence followed by a stretch of poly(U) and/or poly(UC), which varies in size in various HCV strains, and a 98-nucleotide 3'-terminal X region with a fairly conserved sequence (Fig. 9). To determine if these regions play a role in NS3 binding, UV cross-linking studies were carried out with mutant ^{32}P-labeled 3'(+) UTR probes in which either the 98-nucleotide X region (mutant A) (Fig. 9) or the preceding variable sequence and the poly(U) and/or poly(UC) stretch were deleted (mutant B) (Fig. 9). The RNA containing the variable sequence along with the poly(U) and/or poly(UC) stretch showed very little NS3 binding in the absence of the 98-nucleotide X region (mutant A). Similarly, the 98-nucleotide conserved region without the variable and poly(U) and/or poly(UC) sequences was totally defective in NS3 binding (mutant B). When the 3'-terminal SL-I or both (SL-I and -II were deleted from

the entire 3'UTR (mutant C and D; SL-I and SL-I and II), NS3 binding to the 3'(+) UTR was drastically reduced. While these data suggest that SL-I is important, mutant B (with the 98-nucleotide X region only), which still contains SL-I, is also defective in NS3 binding. These results suggest that the presence of the intact 3'-terminal sequences, including the variable region, the poly(U) and/or poly(UC) stretch, and the 98-nucleotide X region, is required for interaction with NS3. The interaction between NS3 and the 3'(+) UTR appears to be specific, since an excess of unlabeled homologous RNA was able to compete out RNA-protein complex formation, whereas a similar-size heterologous RNA was totally inactive in the competition assay. These results suggest that the overall structure (or sequence) of the 3'(+) UTR RNA is required for specific binding of NS3. Additional mutagenesis will be required to clarify the role of the various sequence and/or secondary structural elements within the 3'(+) UTR.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

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We claim:

1. A method for assaying a sample suspected of containing hepatitis C viral (HCV) RNA comprising:

(a) contacting the sample with an NS3 protein, fragment, or mutant thereof to form a nucleoprotein complex; and

(b) measuring the amount or presence of the HCV RNA in the sample.

2. The method of claim 1 wherein the NS3 comprises a label.

3. A kit for assaying a sample suspected of containing a HCV RNA, the kit comprising a composition having an NS3 protein, fragment or mutant thereof.

4. The kit of claim 3 wherein the NS3 comprises a label.

5. The kit of claim 3, further comprising instructions for assaying the sample.

6. An assay system for HCV in a sample suspected of containing HCV RNA comprising:

the sample and an NS3 protein, fragment, or mutant thereof which forms a nucleoprotein complex.

7. The assay system of claim 6 wherein the NS3 comprises a label.

8. A method for inhibiting the binding of helicase to HCV RNA comprising:

contacting the HCV RNA with an NS3 protein, fragment, or mutant thereof to form a nucleoprotein complex, wherein the binding of helicase to the HCV RNA is inhibited.

9. A method for assaying a sample containing a viral RNA comprising:

(a) contacting the sample with a protein, protein fragment, or mutant thereof to form a nucleoprotein complex; and

(b) measuring the amount or presence of the viral RNA that binds to the protein, fragment, or mutant thereof.

10. The method defined in claim 9, wherein the protein, protein fragment or mutant is derived from a protease and/or helicase from a protein, protein fragment or mutant homologous to a protease or helicase.

11. The method defined in claim 10, wherein the protein, protein fragment or mutant is NS3 or 2C.

12. The method defined in claim 9 wherein the viral RNA is positive-stranded RNA virus.

13. The method defined in claim 12 wherein the positive-stranded RNA virus is selected from the group consisting of flavivirus, pestivirus, poliovirus, rhinovirus, and coxsackie virus.

14. A kit for assaying a sample containing a viral RNA comprising:

a protein, protein fragment or mutant thereof that is capable of forming a nucleoprotein in complex with a viral RNA.

15. The kit defined in claim 14, further comprising instructions for use.

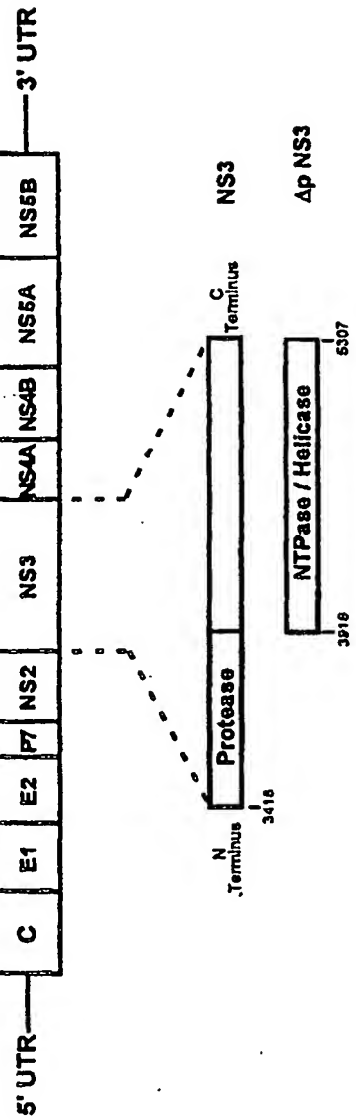
16. A method of treating a subject having HCV infection comprising administering to the subject an agent that inhibits the binding of helicase to HCV RNA.

17. The method of claim 16, wherein the agent is an NS3 protein, fragment or mutant thereof.

18. The method of claim 16, wherein the agent is an antibody.

19. The method of claim 16, wherein the subject is a human.

20. The method of claim 16, wherein the agent is a small molecule chemical compound.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C
E1
E2
P7
NS2
NS3
NS4A
NS4B
NS5A
NS5B
3' UTR
NS3
Δp NS3
C Terminus
N Terminus
Protease
3418
NTPase / Helicase
5307
3918

FIGURE 1

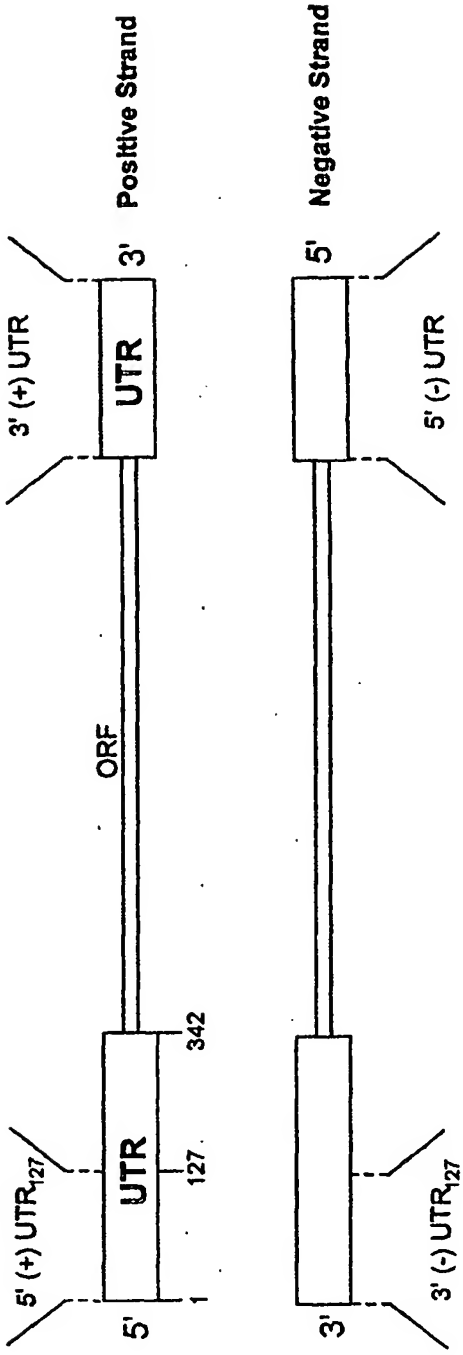
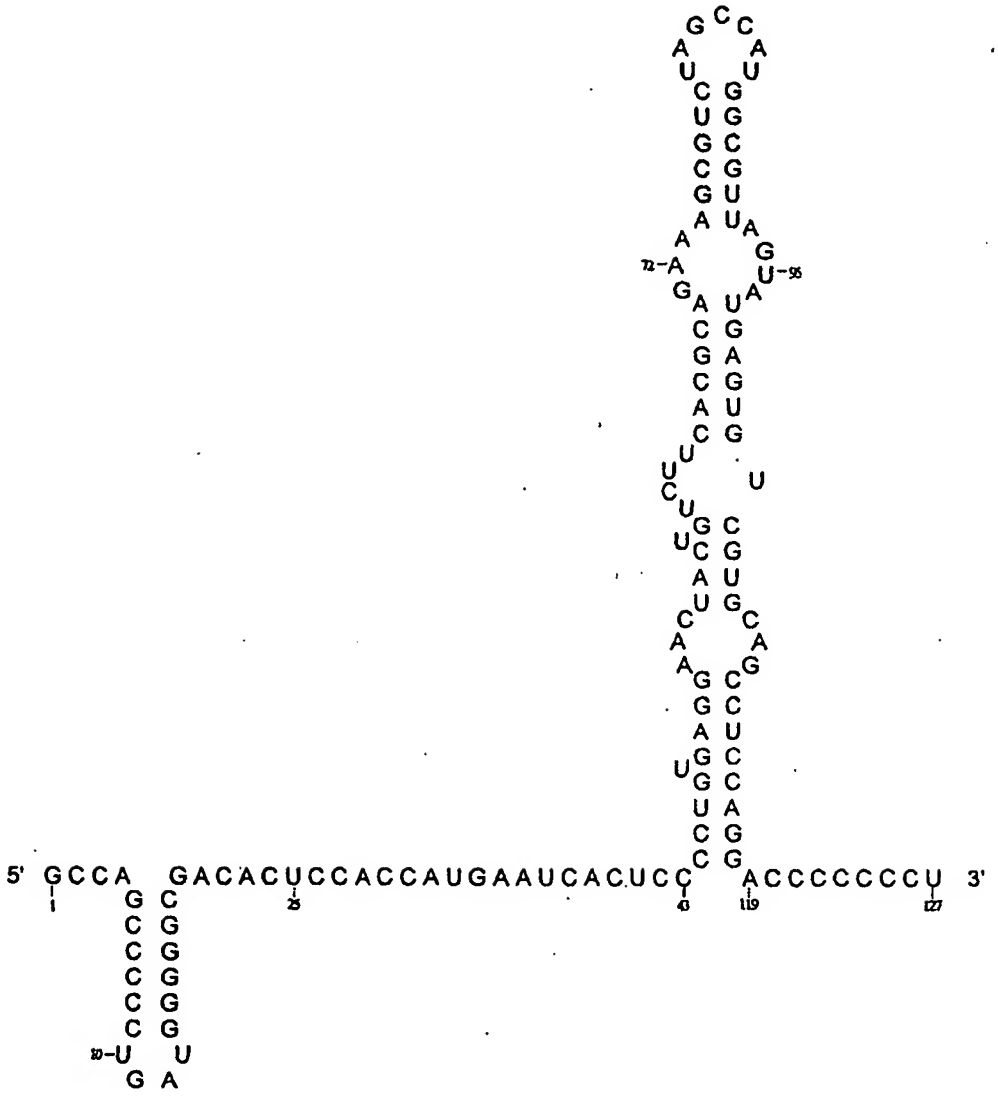

Positive Strand
Negative Strand
3'
5'
3' (+) UTR
UTR
5' (-) UTR
ORF
342
127
1
5' (+) UTR127
UTR
5'
3'
3' (-) UTR127

FIGURE 2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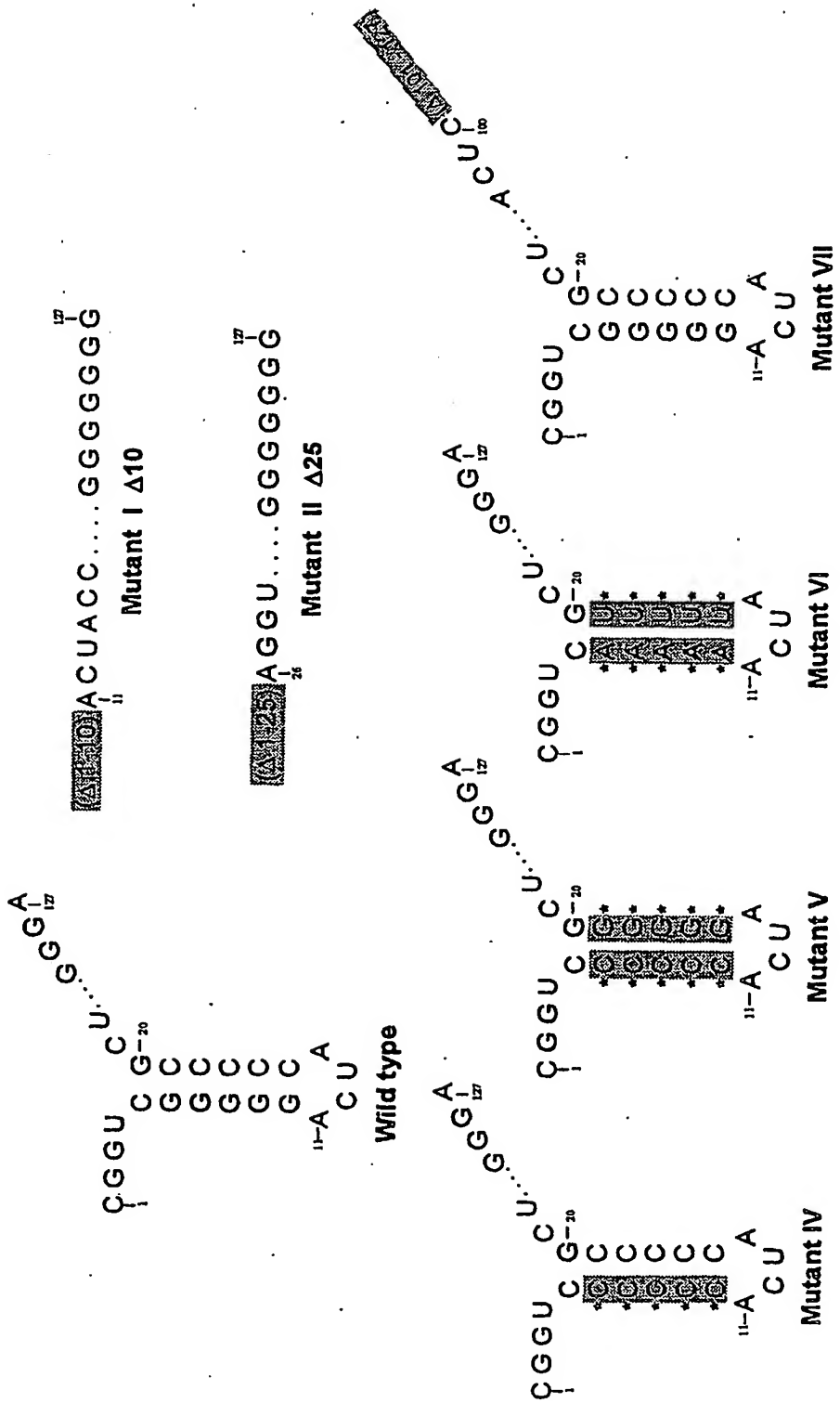
127
72
56
30

FIGURE 3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Mutant II Δ25
Mutant IV
Mutant V
Mutant VI
Mutant VII

FIGURE 4

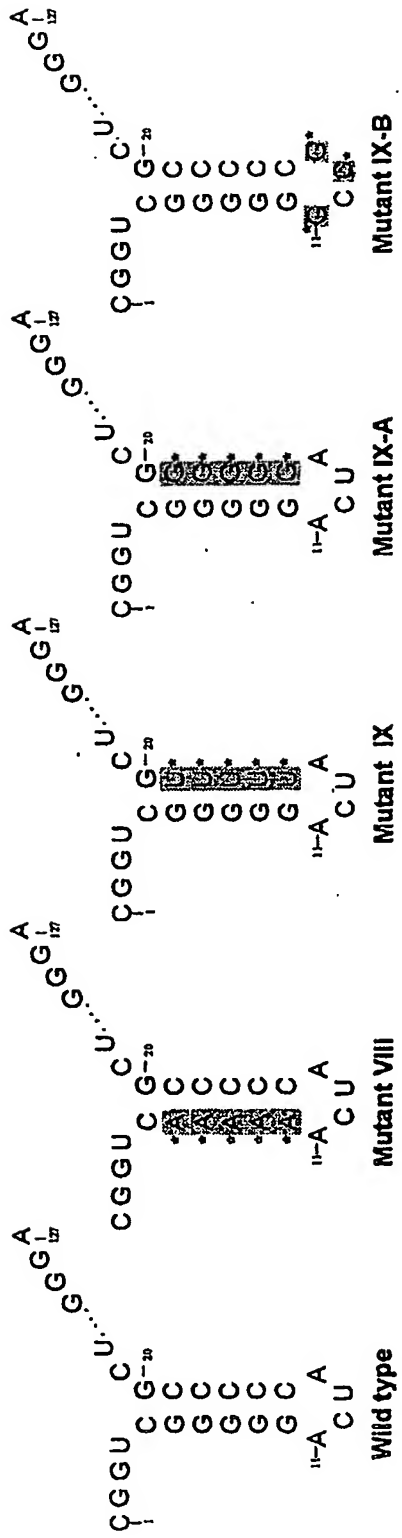


FIGURE 5

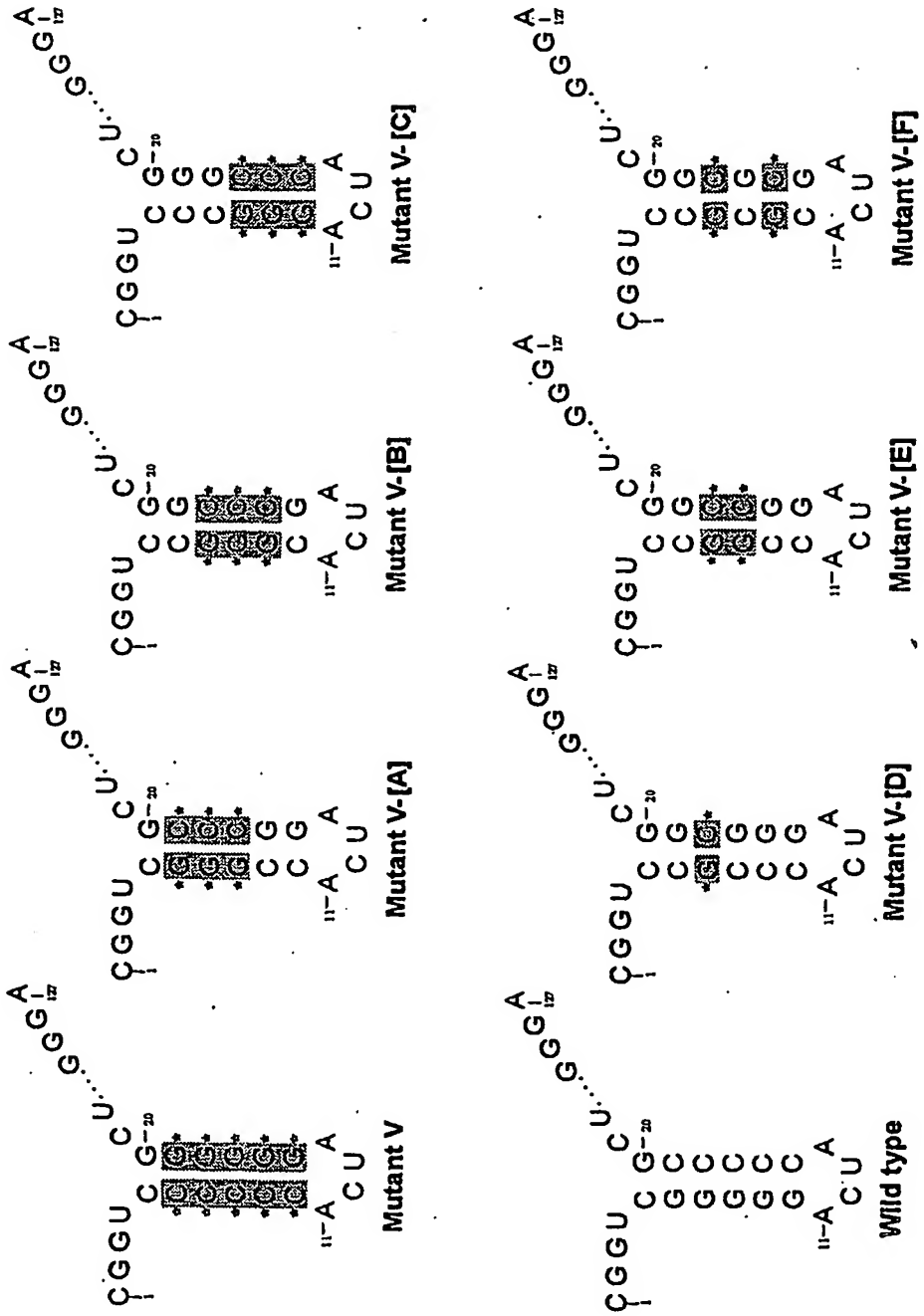

Mutant V
Mutant V-[A]
Mutant V-[B]
Mutant V-[C]
Wild type
Mutant V-[D]
Mutant V-[E]
Mutant V-[F]

FIGURE 6

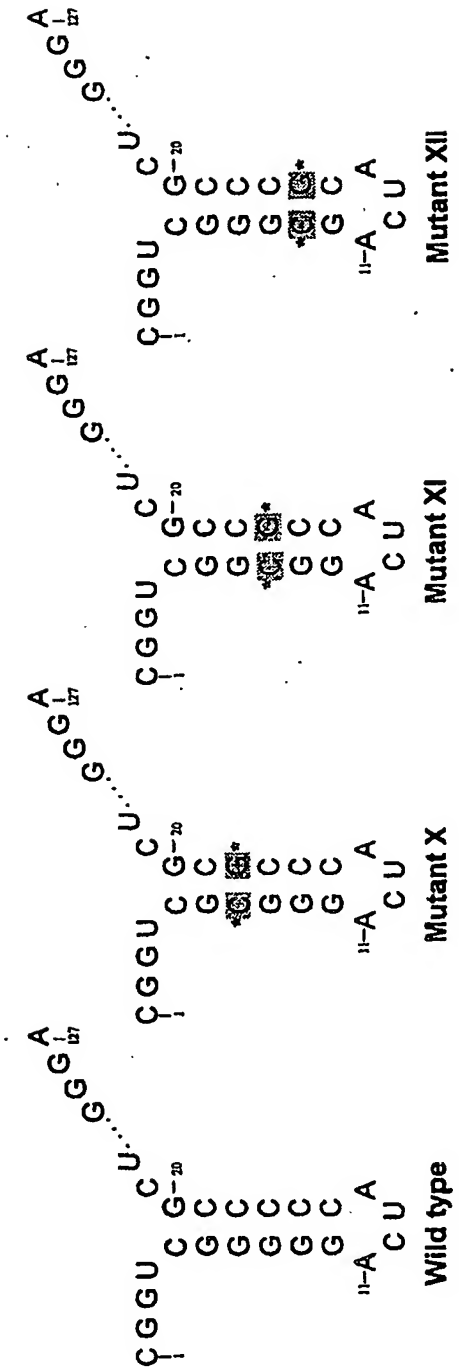


FIGURE 7

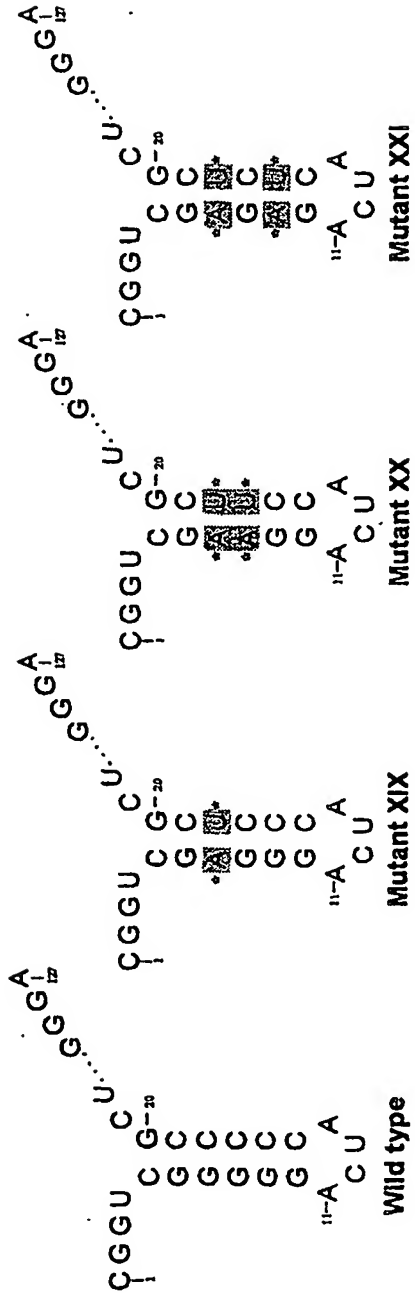
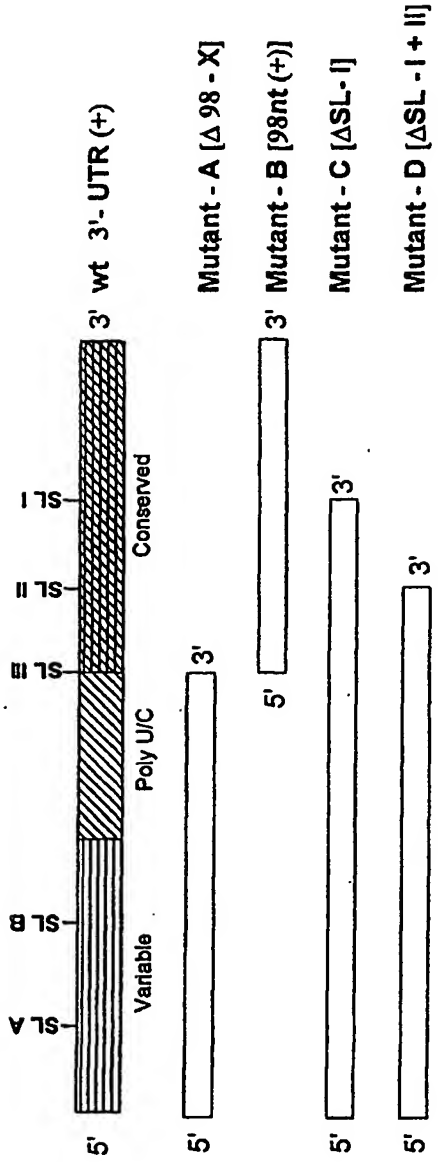


FIGURE 8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SL I
SL II
SL III
SL B
SL A
Conserved
Poly U/C
Variable
5'
Mutant - A [Δ 98 - X]
3'
5'
Mutant - B [98nt (+)]
3'
5'
Mutant - C [ΔSL- I]
3'
5'
Mutant - D [ΔSL - I + II]
3'
5'

FIGURE 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/04916

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/70,1/68,1/34
US CL : 435/5, 6,18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/5, 6,18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/012043 A2 (CHIRON CORPORATION) 03 April 1997, see entire document.	1-7
X	WO 97/027334 A1 (VIROPHARMA INCORPORATED) 31 July 1997, see entire document.	1-7
X	KUMAR et al. Isolation of RNA Aptamers Specific to the NS3 Protein of Hepatitis C Virus from a Pool of Completely Random RNA. Virology 1997, Vol. 237, pages 270-282, especially Fig. 2 and Fig. 3.	1-7
X	GWACK et al. Characterization of RNA Binding Activity and RNA Helicase Activity of the Hepatitis C Virus NS3 Protein. Biochemical and Biopohysical Research Communications. 1996, Vol. 225, pages 654-659, see entire document.	1-7
X	HSU et al. An ELISA for RNA Helicase Activity: Application as an Assay of the NS3 Helicase of Hepatitis C Virus. Biochemical and Biophysical Research Communications. 1998, Vol. 253, pages 594-599, see entire document.	1-7

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2002 (14.06.2002)	13 AUG 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Donna C. Wortman, Ph.D. Telephone No. 703-308-0196

Janice Ford for

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/04916

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GWACK et al. RNA-Stimulated ATPase and RNA Helicase Activities and RNA Binding Domain of Hepatitis G Virus Nonstructural Protein 3. Journal of Virology. Vol. 73, No. 4, pages 2909-2915, see entire document.	1-7, 9-15
X	KLEIN et al. Echovirus-9 protein 2C binds single-stranded RNA unspecifically. Journal of General Virology. 2000, Vol. 81, pages 2481-2484, see entire document.	1-7, 9-15
X	KANAI et al. Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. FEBS Letters. 1995, Vol. 376, pages 221-224, see entire document.	1-7, 9-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/04916

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-7 and 9-15

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/04916

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-7 and 9-15, drawn to a method and kit for assaying for HCV RNA.

Group II, claim 8, drawn to a method of inhibiting helicase binding.

Group III, claims 16, 17, and 19, drawn to a method of treating using a protein.

Group IV, claims 16, 18, and 19, drawn to a method of treating using an antibody.

Group V, claims 16, 19, and 20, drawn to a method of treating using a small molecule.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of Group I are drawn to a method for assaying for HCV RNA and compositions for use in the method. The claims of Groups II-IV are drawn to methods that do not require the compositions of Group I and/or require different process steps and/or have different goals and outcomes from the method of Group I. PCT Rule 13 does not provide for multiple processes.